The Effects of Drugs, Other Foreign Compounds, and Cigarette Smoke on the Synthesis of Protein by Lung Slices

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The incorporation of ^{14}C-leucine into rabbit lung slices was monitored in the absence and presence of selected drugs and chemicals relevant to the perturbation of lung function and the development of lung disease. Known inhibitors of protein synthesis (cycloheximide and ricin) inhibited the incorporation of ^{14}C-leucine. Marked inhibition was also recorded with the lung toxins paraquat and 4-ipomeanol. By contrast, orciprenaline, salbutamol, and terbutaline were without effect although some response was recorded with isoprenaline. The filtered gas phase of cigarette smoke and acrolein, one of its components, were inhibitory but protection was afforded by *N*-acetylcysteine. It is suggested that the inhibitory effects of cigarette smoke may be due to its acrolein content. It is further suggested that the use of lung slices and measurements of ^{14}C-leucine incorporation provide valuable means for monitoring potential pulmonary toxins.

Introduction

The chemical challenge to the lungs by airborne compounds and agents is formidable, and the capability and capacity of pulmonary tissue in the detoxication of noxious chemicals has been extensively investigated (*1*,*2*). It is also well known that the lung is particularly vulnerable to certain toxic chemicals. For example, paraquat (*3*) and 4-ipomeanol (*4*,*5*) are known to cause particularly severe and specific damage to lung tissue. Although the eventual response to various toxic chemicals in terms of pulmonary disease has been described, much remains to be learned about cellular metabolic activity and overall lung function and the mechanisms by which intermediary metabolism is altered in disease.

It might be expected that perturbations of pulmonary intermediary metabolism caused by noxious chemicals would be reflected in the relative efficiencies of the defense mechanisms. This study seeks to investigate those perturbations and to design a model system for monitoring the initial effects of various chemicals on the lung.

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The synthesis of protein by the lung was studied in the presence of selected drugs and chemicals that are known to be pertinent to lung function and to lung disease. Protein biosynthesis was chosen as an indicator of initial toxic effect because the formation of protein requires the integration and cooperation of the many pathways culminating in amino acid assembly. Alterations in the rates of any one of those pathways would be reflected in the rate of protein biosynthesis.

A range of compounds was studied, and included those known to cause pulmonary conditions such as edema, fibrosis, and hemorrhage; and several bronchiodilators normally administered directly to the lungs by inhalation. These included the selective β_2-adrenoceptor stimulants salbutamol and terbutaline, the nonselective β-adrenoceptor stimulants isoprenaline and orciprenaline, and the corticosteroid betamethasone. In addition, the effects were studied of the filtered gas phase of cigarette smoke and acrolein, one of its components.

Materials and Methods

Chemicals

[U^{14}C]Leucine (37 mCi/mmole), PCS scintillation fluid, and a NCS tissue solubilizer were purchased from the Radiochemical Centre (Amersham, Bucks, UK).

MEM Eagles medium was purchased from Gibco Ltd. (Paisley, Scotland); ricin and *N*-acetylcysteine from Sigma Chemical Co. (Poole, Dorset, UK); paraquat from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, UK); and acrolein from Fluka AG (Chemische Fabrik CH-9470 Buchs). Salbutamol, isoprenaline, terbutaline, orciprenaline, betamethasone sodium phosphate (Becotide), bleomycin, and cycloheximide were obtained from Dr. B. Evans (Department of Pharmacy, University of Wales College of Medicine, UK) and 4-ipomeanol was a gift from M. R. Boyd (National Cancer Institute, MD). All other chemicals were obtained from British Drug Houses (Poole, Dorset, UK).

Cigarette Smoke

Freshly drawn filtered cigarette smoke was used in all experiments and was prepared as described by Green and Carolin (*6*). Filter tips were removed from the cigarettes before smoking. Aqueous extracts of the filtered gas phase of cigarette smoke were prepared in modified Krebs-Ringer phosphate medium by drawing 25 mL portions of smoke into syringes containing 1 mL of the medium.

Animals

Mature New Zealand White rabbits (2.5–3.5 kg) maintained on a standard diet (rabbit pellets, Pilsbury's Ltd., Priory Road, Edgbaston, Birmingham, UK) were used throughout.

Preparation of Lung Slices

Rabbits were anesthetized with Sagatal (60 mg/kg) administered via the marginal ear vein. Heparin (1000 units/kg) was then injected. Following a midline abdominal incision, animals were exsanguinated by cannulation of the abdominal aorta toward the heart. The thoracic cavity was opened after the diaphragm had been punctured, and a cannula was inserted into the pulmonary artery via the left auricle and secured with a ligature. Isotonic saline (0.9% wv, ~ 50 mL at 37°C) was then infused, and the heart was incised to allow exit of the saline solution. The trachea was then isolated, clamped, and the heart-lung preparation was removed. The preparation was washed briefly with warm (37°C) isotonic saline, and the heart, together with other tissue, was dissected from the lungs that were then washed in phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4, at 4°C). The trachea was removed and lung slices (~0.5 mm), cut manually using a razor blade on a cooled (4°C) glass plate, were placed in phosphate-buffered saline (4°C).

Measurement of Protein Production by Lung Slices

Lung slices (~250 mg wet wt) were rinsed in the incubation medium [1 vol of MEM Eagles medium: 9 vol of water: 10 vol of phosphate-buffered saline, 0.5 mM ascorbic acid, adjusted to pH 7.4 (with $NaHCO_3$, 4.4% w/v) and equilibrated with a gas mixture (95% O_2/5% CO_2)]. The slices were incubated for two successive periods (preincubation and incubation periods). In the preincubation period, slices were incubated in vessels containing 1 mL of the gassed medium and maintained at 37°C for 45 min. The vessels were shaken.

In all experiments except for some with ricin, the compounds under investigation were present in the preincubation period only. In experiments using the filtered gas phase of cigarette smoke, lung slices were preincubated with 1 mL of a modified Krebs-Ringers phosphate medium containing the smoke and prepared as described previously.

After the preincubation period, the medium was aspirated and discarded, and fresh medium (1 mL) containing ^{14}C-leucine (0.2 μCi/mL) was added. Vessels were then gassed with an oxygen-carbon dioxide mixture (95:5 v/v) and incubated at 37°C, with shaking, for periods ranging from 15 min to 3 hr. At the end of the incubation period the lung slices were separated from media by vacuum-assisted filtering and rinsed (3 times with 10 mL phosphate-buffered saline at 4°C). Lung slices were then homogenized (Tri-R instrument, Rockville Center, NY) in 2.5 mL of phosphate-buffered saline and perchloric acid (0.5 mL, 1 M) was added immediately. After 30 min at room temperature the homogenates were centrifuged (600*g* for 20 min). Pellets were resuspended in 2 mL of water and 0.5 mL of 1 M perchloric acid followed by centrifugation (600*g* for 20 min). This procedure was repeated three times, and the pellets were then resuspended in 1 mL of methanol followed by centrifuging (600*g* for 10 min). Supernatants were removed and pellets were dried at 50°C for 16 hr. Each pellet was then crushed and divided into two portions. One portion was assayed for protein content by the method of Lowry et al. (*7*) using bovine serum albumin as the standard. The other portion was used for assaying the incorporation of ^{14}C-leucine. It was incubated at 50°C for 4 hr with 1 mL of NCS tissue solubilizer. After cooling, solutions were bleached by adding 0.6 mL of H_2O_2 (30%, w/v) in 0.2 mL portions and 0.2 mL propan-2-ol. Solutions were then heated (50°C) for 10 min, and after cooling they were stored in the dark for 16 hr. Glacial acetic acid (1 mL) and PCS (15 mL) were added to each sample, and the radioactive contents were determined by liquid scintillation counting (LKB 1217 Rackbeta liquid scintillation counter).

Results

Measurement of Protein Production by Lung Slices in the Presence of Foreign Compounds

In most experiments test compounds were present in the preincubation period only. However, in some

experiments they were present in the incubation period or in both the preincubation and incubation periods.

In initial experiments the rate of protein production was measured in lung slices under control conditions in the absence of potential toxins. After an initial lag phase of approximately 10 to 15 min, there was linear incorporation of radioactivity into protein, and this was maintained over 3 hr. Incorporation rates ranged from 2.22 $\times$ 10^{-4} μCi/mg protein/3 hr to 22.2 $\times$ 10^{-4} μCi/mg protein/3 hr in 35 separate experiments; in 70% of the experiments the incorporation rate was in the range 3 to 7 $\times$ 10^{-4} μCi/mg protein/3 hr. In all experiments in which the effects of potential toxins were assessed, the rates of incorporation of radioactivity in the absence of toxins were also measured.

The experimental system was tested in the presence of known inhibitors of protein synthesis, namely, cycloheximide and ricin. Lung slices were incubated with cycloheximide at two concentrations (0.5 and 1 g/mM during the preincubation period only). It was shown that protein synthesis was inhibited almost completely, with the incorporation of ^{14}C-leucine reduced to 3.2% of the control value at both concentrations of cycloheximide.

Protein synthesis was also severely inhibited by ricin. The effect of ricin at two concentrations (4 μg/mg/mL and 10 μmL) added to lung slices after the preincubation period is illustrated in Figure 1. The rates of incorporation of ^{14}C-leucine in the presence of ricin were identical to controls until 1 hr of incubation, but thereafter there was virtually no incorporation.

In other experiments, ricin at the same concentrations (4 and 10 μg/mL) was present during the preincubation period only. Test and control ^{14}C incorporation figures were virtually indistinguishable after 30 min of incubation, but thereafter—although protein production proceeded in the presence of ricin and the rate was linear—it was reduced to approximately 20% of the control rate at both concentrations of inhibitor. The results of experiments in which the incorporation of ^{14}C was measured in the presence of various concentrations of ricin present during the preincubation period only are shown in Figure 2 and demonstrate that the inhibition is dose related.

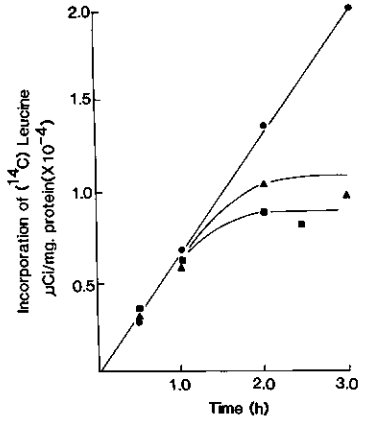

FIGURE 1. Incorporation of ^{14}C-leucine into protein by lung slices in the absence of toxin (●) and in the presence of ricin at two concentrations, 4 μg/mL (▲) and 10 μg/mL (■). Ricin was present in the incubation period only, i.e., added at time zero. Each point represents the mean of two determinations.

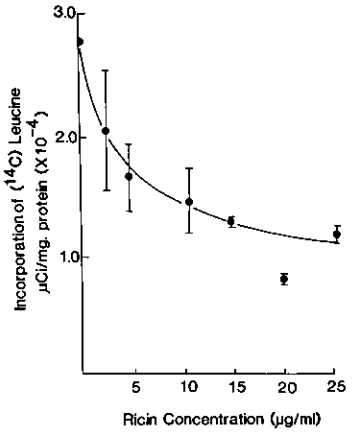

FIGURE 2. The effect of ricin concentration on the incorporation of ^{14}C-leucine into protein over 3 hr. The ricin was present in the preincubation period only. Each point represents the mean of two determinations. Range values are shown by vertical lines.

Experiments with Selected Drugs

In these experiments the concentrations of drugs were based on clinical dose regimes. Thus, the nonselective β-receptor stimulants isoprenaline and orciprenaline were incubated with lung slices at concentrations ranging from 0.074 to 0.93 mM and 0.11 to 10.60 mM, respectively. Some inhibition (Table 1) of ^{14}C-leucine incorporation was recorded with isoprenaline at higher concentrations, but with orciprenaline the rate of incorporation was higher than control values at all concentrations of the drug. The results obtained with the more selective β_2-receptor stimulants terbutaline and salbutamol are also shown in Table 1; it is evident that neither drug affected significantly the incorporation of ^{14}C-leucine. When the corticosteroid betamethasone was present in lung slice incubates, a biphasic response was noted. Thus, when the drug was present at high concentrations (0.81 mM and 0.62 mM) incorporation was inhibited by approximately 50%, whereas at a concentration of 0.11 mM there was stimulation of ^{14}C incorporation by 66%.

Experiments with Pulmonary Toxins

The effects on protein synthesis of the established pulmonary toxins paraquat and 4-ipomeanol were measured at the concentration ranges of 0.1 to 1.0 mM and 0.06 to 0.65 mM, respectively. Inhibition of protein synthesis was recorded at all concentrations of the compounds and the inhibition was dose related (Fig. 3).

Table 1. The effects of some bronchodilatory drugs on the incorporation of ^{14}C-leucine into protein in lung slices.[a]

Drug	Concentration, mM	Incorporation of ^{14}C-leucine into lung protein over 3 hr, % of control
Isoprenaline	0.93	85
	0.67	84
	0.37	96
	0.22	84
	0.074	108
Orciprenaline	10.60	112
	5.30	115
	2.10	129
	0.53	114
	0.11	142
Terbutaline	4.39	99
	2.63	125
	1.00	104
	0.20	135
	0.10	110
Salbutamol	4.34	109
	0.09	112
Betamethasone	0.81	48
	0.62	48
	0.43	76
	0.22	120

[a]Each figure is the average of two separate incubations.

Experiments with Cigarette Smoke and Acrolein

In all experiments the incorporation of ^{14}C-leucine was inhibited by aqueous extracts of the filtered gas phase (25 mL or 50 mL) of cigarette smoke. The results of a typical experiment in which incorporation was greatly reduced in the presence of 50 mL of smoke is shown in Figure 4. Protection from the effects of smoke was provided when *N*-acetylcysteine (0.7 mM) was incorporated into the medium before exposure to smoke (Fig. 4).

The effect of acrolein on the incorporation of ^{14}C-leucine over 3 hr was measured at acrolein concentrations ranging from 0.038 mM to 0.38 mM. In all experiments a biphasic response was recorded as

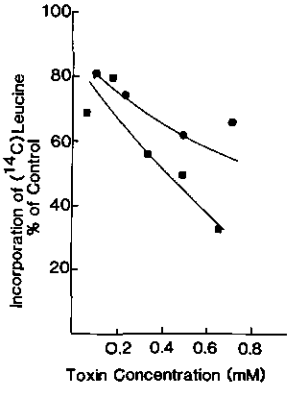

FIGURE 3. The effect of paraquat (●) and 4-ipomeanol (■) on the incorporation of ^{14}C-leucine into protein over 3 hr. Toxins were present in the preincubation period only. Each point represents the mean of two determinations (no range, but present on other graphs, e.g., Figs. 2–5).

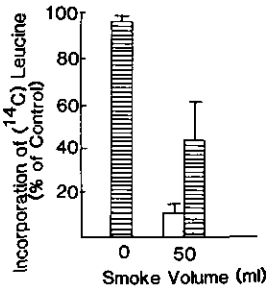

FIGURE 4. The effect of the filtered gas phase of cigarette smoke on the incorporation of ^{14}C-leucine into protein over 3 hr in the absence (□) and presence (▤) of *N*-acetylcysteine (0.7 mM). Smoke and/or *N*-acetylcysteine were present in the preincubation period only. Each value is the mean of two determinations. Range values are shown by vertical lines.

illustrated in Figure 5. At concentrations of 0.038 mM and 0.076 mM there were slight increases in the rates of incorporation, but at higher concentrations the incorporation was markedly inhibited. Further time-course studies (Fig. 6) with inhibitory concentrations of acrolein (0.17 mM, 0.36 mM, and 0.89 mM) demonstrated that although the incorporation of ^{14}C-leucine was markedly reduced at each concentration, the incorporation rates were linear. With acrolein at 0.89 mM, inhibition was virtually complete.

Further experiments with acrolein were carried out in which ^{14}C-leucine incorporation was measured over 3 hr in lung slices incubated with acrolein at two concentrations (0.21 mM and 0.36 mM) in the absence and presence of *N*-acetylcysteine (0.7 mM) (Fig. 7). The incorporation of ^{14}C-leucine was inhibited by 31% and 63%, respectively, but no inhibition was recorded when *N*-acetylcysteine was present in the media.

Discussion

The list of new compounds and agents to which the lungs are subjected is enormous, yet, in very few cases have studies been made on the interactions between these agents and the lungs. In this study we have described an experimental method for measuring the effects of potential pulmonary toxins on the lungs, and we have

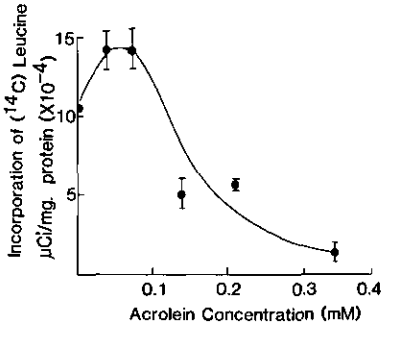

FIGURE 5. The effect of acrolein concentration on the incorporation of ^{14}C-leucine into protein by slices. Each value is the mean of two determinations. Range values shown by vertical lines.

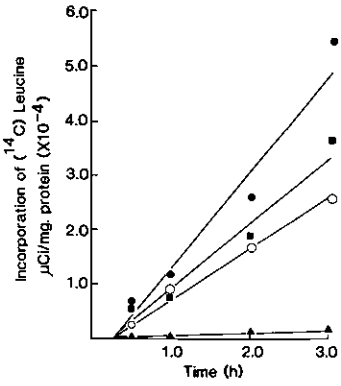

FIGURE 6. The effect of various concentrations of acrolein (■ = 0.17 mM; 0 = 0.36 mM; ▲ = 0.89 mM) on the rate of incorporation of ^{14}C-leucine into protein by lung slices (● = control).

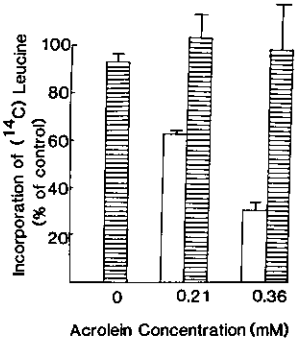

FIGURE 7. The effect of acrolein on the incorporation of ^{14}C-leucine over 3 hr in the absence (□) and presence (▤) of *N*-acetylcysteine. The acrolein and *N*-acetylcysteine were present in the preincubation only. Each value is the mean of two determinations. Range values are shown by vertical lines.

demonstrated that protein biosynthesis by lung slices can be used as a valuable indicator of the interactions between chemicals and pulmonary metabolism. It was shown that two known inhibitors of protein synthesis, ricin and cycloheximide, were potent inhibitors at low concentrations and, furthermore, that ricin inhibition was dose dependent.

In all experiments involving drugs as test compounds, the concentration ranges were compiled from the dose regimes used clinically. It is noteworthy that quite different results were obtained with the nonselective β-receptor stimulants isoprenaline and orciprenaline. Although orciprenaline was used at approximately 10 times the concentration of isoprenaline, the drug had no adverse effects on protein biosynthesis, and the enhancement of ^{14}C-leucine incorporation was recorded at all concentrations of the drug. By contrast, isoprenaline was inhibitory at the higher concentrations used. Neither terbutaline nor salbutamol was inhibitory, and, as with orciprenaline, stimulation of ^{14}C-leucine incorporation was recorded at some dose levels. Betamethasone, a glucocorticoid, on the other hand, showed a biphasic action; at the lowest dosage the incorporation levels were significantly enhanced while at the highest dosages there was more than 50% inhibition.

Collectively, the results obtained with drugs used clinically illustrate that some drugs have no adverse effects on the numerous biochemical transformations culminating in amino acid assembly, while others are markedly inhibitory. In the case of inhibitory drugs, a number of new avenues are opened. For example, it may be important to determine whether inhibition of protein synthesis is selective or not. The possibility is also presented of modifying the drug structure so that therapeutic potency is preserved but inhibitory properties are abolished.

The involvement of cigarette smoke in lung disease has been much studied, and several workers have questioned the significance of the acrolein content of smoke (*8*). In this study we report the inhibitory effects of both the aqueous extract of cigarette smoke and acrolein in the synthesis of protein, and the dose regime was chosen to be in the same range as that present in cigarette smoke [~ 4.1 μg acrolein/10 mL filtred smoke (*8*)]. Acrolein is a well-known cytotoxin that is widely distributed in biological material (*9*). In an early study Willmer and Walersteiner (*10*) showed that various aldehydes inhibited growth and cellular activity in the concentration region of 2 mM. However, acrolein produced similar inhibitory effects at considerably lower concentrations (20 μM). In addition to the importance of the presence of acrolein in cigarette smoke, this aldehyde is widely used in industry as a synthetic intermediate and is evolved during the overheating of biological material (*11*). Acrolein has also been shown to be the ultimate metabolite responsible for the observed toxicity of several compounds including endogenous polyamines, spermine, and spermidine (*12*) that are ubiquitous in animal tissues (*13*), and allyl alcohol (*14*). Recent studies implicate acrolein to be a mediator of the pulmonary (*15*) and renal (*16*) toxicity of cyclophosphamide that is extensively used in therapy as an antineoplastic and immunosuppressive agent. It has also been suggested that the antineoplastic effects of spermine (*17*) and cyclophosphamide (*18*) may be, in part, due to the liberation of acrolein *in situ*. Thus, the inhibitory effect of acrolein on protein biosynthesis may have far-reaching significance. As with the compounds discussed previously, it may well be important to study the specificity of action with respect to the synthesis and the half-lives of proteins whose synthesis is affected.

Attempts have been made to relate the effects of aqueous cigarette smoke and acrolein on the incorporation of amino acids into alveolar macrophage protein, and it has been suggested (*8*) that the effects are quantitatively and qualitatively different. However, experience in the present investigation suggests that great care must be exercised in interpreting the effects of acrolein at low concentrations because of the volatility

and instability of the aldehyde. Also when evaluating the effects of acrolein in terms of its affinity for -SH groups, by experiments in the presence of *N*-acetylcysteine, it is essential that *N*-acetylcysteine is added before acrolein because the effects of acrolein are not reversible. It may well be that conflicting reports on protection from acrolein by *N*-acetylcysteine (*19,20*) can be explained in terms of the experimental protocol. A preliminary account of the present work has been presented previously (*21*).

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